SECTION 11

TOPSMELT, *Atherinops affinis*
7-DAY LARVAL GROWTH AND SURVIVAL TEST METHOD

Adapted from a method developed by
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TOPSMELT, *ATHERINOPS AFFINIS*
LARVAL SURVIVAL AND GROWTH TEST

11.1 SCOPE AND APPLICATION

11.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the topsmelt, *Atherinops affinis*, using nine-to-fifteen day old larvae in a seven-day, static-renewal exposure test. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological an biochemical functions of the test organisms.

11.1.2 Daily observations of mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

11.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

11.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

11.1.5 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

11.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

11.2 SUMMARY OF METHOD

11.2.1 This method provides step-by-step instructions for performing a 7-day static-renewal toxicity test using survival and growth of topsmelt larval fish to determine the toxicity of substances in marine and estuarine waters. The test endpoints are survival and growth.

1.3 INTERFERENCES

11.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

11.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

11.3.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

11.3.4 Food added during the test may sequester metals and other toxic substances and confound test results.

11.4 SAFETY

11.4.1 See Section 3, Health and Safety.

11.5 APPARATUS AND EQUIPMENT

11.5.1 Tanks, trays, or aquaria -- for holding and acclimating topsmelt, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system. (See Anderson et al., 1994, Middaugh and Anderson, 1993).

11.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

11.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, and larvae at test temperature (20EC) prior to the test.

11.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

11.5.5 Refractometer -- for determining salinity.

11.5.6 Hydrometer(s) -- for calibrating refractometer.

11.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

11.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

11.5.9 pH and DO meters -- for routine physical and chemical measurements.

11.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

11.5.11 Winkler bottles -- for dissolved oxygen determinations.

11.5.12 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

11.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

11.5.14 Glass stirring rods -- for mixing test solutions.

11.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

11.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

11.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

11.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

11.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

11.5.20 Wash bottles -- for dilution water.

11.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

11.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

11.5.23 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

11.5.24 Brine shrimp, *Artemia*, culture unit -- see Subsection 11.6.25 and Section 4, Quality Assurance.

11.5.25 Separatory funnels, 2-L -- two-four for culturing *Artemia*.

11.5.26 Siphon tubes (fire polished glass) -- for solution renewals and handling larval fish.

11.5.27 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

11.5.28 Siphon with bulb and clamp -- for cleaning test chambers.

11.5.29 Light box -- for counting and observing larvae.

11.5.30 White plastic tray -- for collecting larvae during cleaning of the test chambers.

11.5.31 Forceps -- for transferring dried larvae to weighing pans.

11.5.32 Desiccator -- for holding dried larvae.

11.5.33 Drying oven -- 50-105EC range, for drying larvae.

11.5.34 NITEX® mesh screen tubes - (#150 µm, 500 µm, 3 to 5 mm) -- for collecting *Artemia* nauplii and fish larvae. (NITEX® is available from Sterling Marine Products, 18 Label Street, Montclair, NJ 07042; 201-783-9800).

11.5.35 60 µm Nitex® filter -- for filtering receiving water.

11.6 REAGENTS AND SUPPLIES

11.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

11.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

11.6.3 Tape, colored -- for labelling test chambers and containers.

11.6.4 Markers, water-proof -- for marking containers, etc.

11.6.5 Parafilm -- to cover graduated cylinders and vessels.

11.6.6 Gloves, disposable -- for personal protection from contamination.

11.6.7 Pipets, serological -- l-l0 mL, graduated.

11.6.8 Pipet tips -- for automatic pipets.

11.6.9 Coverslips -- for microscope slides.

11.6.10 Lens paper -- for cleaning microscope optics.

11.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

11.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

11.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

11.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

11.6.15 Laboratory quality assurance samples and standards -- for the above methods.

11.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

11.6.17 Ethanol (70%) or formalin (4%) -- for preserving the larvae.

11.6.18 *Artemia* nauplii -- for feeding test organisms.

11.6.19 Weigh boats or weighing paper -- for weighing reference toxicants.

11.6.20 Reference toxicant solutions (see Subsection 11.10.2.4 and see Section 4, Quality Assurance).

11.6.21 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

11.6.22 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

11.6.23 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 11.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

11.6.24 HYPERSALINE BRINES

11.6.24.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

11.6.24.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

11.6.24.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

11.6.24.4 Freeze Preparation of Brine

11.6.24.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

11.6.24.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

11.6.24.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

11.6.24.5 Heat Preparation of Brine

11.6.24.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

11.6.24.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

11.6.24.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

11.6.24.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

11.6.24.6 Artificial Sea Salts

11.6.24.6.1 No data from topsmelt larval tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

11.6.24.7 Dilution Water Preparation from Brine

11.6.24.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

11.6.24.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part plus 1.94 reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

11.6.24.8 Test Solution Salinity Adjustment

11.6.24.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

11.6.24.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see subsection 8.8.9, Effluent and Receiving Water Sampling, Sampling Handling, and Sample Preparation for Toxicity Tests).

11.6.24.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

11.6.24.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

11.6.24.9 Preparing Test Solutions

11.6.24.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

11.6.24.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine(68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

11.6.24.10 Brine Controls

11.6.24.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See SubSection, 11.6.24.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

11.6.25 BRINE SHRIMP, *ARTEMIA SP*., NAUPLII -- for feeding cultures and test organisms.

11.6.25.1 Newly hatched *Artemia* sp. nauplii are used for food for the test organisms. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size. (One source that has been found to be acceptable is Aquarium Products, 180L Penrod Ct., Glen Burnie, Maryland 21061). For commercial sources of brine shrimp, *Artemia*, cysts, see Table 2 of Section 5, Facilities, Equipment, and Supplies); and Section 4, Quality Assurance.

11.6.25.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts, or may be obtained from the Quality Assurance Research Division, EMSL, Cincinnati, OH 45268, 513-569-7325. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine pesticides 0.15 ug/g wet weight or that the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight (For analytical methods see USEPA, 1982).

11.6.25.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or an aqueous unionized salt (NaCl) solution prepared with 35 g salt or artificial sea salts per liter, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27EC. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985a; USEPA, 1993a; ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes with a dark cloth or paper towel. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a funnel fitted with a #150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

11.6.25.4 Testing *Artemia* nauplii as food for toxicity test organisms.

11.6.25.4.1 The primary criteria for acceptability of each new supply of brine shrimp cysts is adequate survival, and growth of the larvae. The larvae used to evaluate the acceptability of the brine shrimp nauplii must be the same geographical origin and stage of development (9 to 15 days old) as those used routinely in the toxicity tests. Two 7-day chronic tests are performed side-by-side, each consisting of five replicate test vessels containing five larvae (25 organisms per test, total of 50 organisms). The juveniles in one set of test chambers is fed reference (acceptable) nauplii and the other set is fed nauplii from the "new" source of *Artemia* cysts.

11.6.25.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the tests, and age of the *Artemia* nauplii at the start of the test, should be the same as used for the routine toxicity tests.

11.6.25.4.3 Results of the brine shrimp, *Artemia*, nauplii nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival or growth of the mysids fed the two sources of nauplii.

11.6.26 TEST ORGANISMS

11.6.26.1 The test organisms for test method are larvae of the topsmelt, *Atherinops affinis*. Topsmelt occur from the Gulf of California to Vancouver Island, British Columbia (Miller and Lea, 1972). It is often among the most abundant fish species in central and southern California estuaries (Allen and Horn, 1975; Horn, 1979; Allen, 1982). Topsmelt reproduce from May through August, depositing eggs on benthic algae in the upper ends of estuaries and bays (Croaker, 1934; Fronk, 1969). Off-season spawning of *Atherinops affinis* has been successful in a laboratory-held population (Anderson et al., 1994). Their embryonic development is similar to that of other atherinids used widely in toxicity testing (eg, *Menidia* species, Borthwick et al.,1985; Middaugh et al., 1987; Middaugh and Shenker, 1988), and methods to assess sublethal effects with these species have proven to be adaptable for topsmelt (Anderson et al., 1991, Middaugh and Anderson, 1993, McNulty et al., 1994).

11.6.26.2 Species Identification

11.6.26.2.1 Topsmelt often co-occur with jacksmelt, *Atherinopsis californiensis*. The two species can be distinguished based on several key characteristics. Jacksmelt have 10-12 scales between their two dorsal fins; topsmelt have 5-8 scales between the two fins. Jacksmelt teeth are arranged in several bands on each jaw and the teeth are not forked; topsmelt teeth are arranged in one band and the teeth are forked. In jacksmelt, the insertion of the first dorsal fin occurs well in advance of the origin of the anal fin. In topsmelt, the origin of the anal fin is under the insertion of the first dorsal fin. Consult Miller and Lea (1972) for a guide to the taxonomy of these two fishes.

11.6.26.3 Obtaining Broodstock

11.6.26.3.1 In California, adult topsmelt can be seined from sandy beaches in sloughs and estuaries from April through August. The size of the seine used depends on the number of people deploying it and the habitat being sampled. Larger seines can be used in open sandy areas, smaller seines are used in smaller areas with rocky outcroppings. Five or six people are an adequate number to set and haul a 100-ft beach seine. The seine is set on an ebbing tide using a small motor skiff with one person driving and a second deploying the net from the bow. The net is set parallel to shore then hauled in evenly from the wings. The net mesh diameter should be small enough to prevent the fish from damaging themselves; a one-centimeter diameter mesh in the middle panel and one-and-a-half-centimeter diameter mesh in the wing panel is adequate. As the net is pulled onto the shore, the adult topsmelt are sorted into five-liter plastic buckets, then immediately transferred to 100-liter transport tanks.

11.6.26.3.2 State collection permits are usually required for collection of topsmelt. Collection is prohibited or restricted in some areas. Collection of topsmelt is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of fish broodstock.

11.6.26.3.3 Various containers can be used to transport fish; 100-liter covered plastic trash cans have been used successfully to transport topsmelt. New plastic containers should be leached in seawater for 96 hours prior to transporting fish. Each container can maintain approximately 20 adult fish for six to eight hours if adequate aeration is provided. Use compressed oxygen or air to supply aeration to the tanks during transport.

11.6.26.4 Broodstock Culture and Handling

11.6.26.4.1 Once in the laboratory the fish should be treated for 2 days with a general antibiotic in a separate tank (eg., Prefuran® as per label instructions), then divided among 1000-liter holding tanks. No more than 30 adult fish should be placed in each tank. Tank temperature should be maintained at 18EC using a 1500-watt immersion heater. To conserve heated seawater, the seawater in the tanks can be recirculated using the system similar to that described by Middaugh and Hemmer (1984). A one-thirtieth (1/30)-hp electric pump is used to circulate water (10 liters/minute) from the tanks through vertical, biologically activated nylon filter elements located in a separate reservoir, then back into the tanks. Fresh seawater should be constantly provided to the system at 0.5 liters/minute to supplement the recirculated seawater. The tanks are insulated with one inch thick closed cell foam to conserve heat. Dissolved oxygen levels should be maintained at greater than 6.0 mg/liter using aeration. Salinity should be checked periodically using a refractometer accurate to the nearest 0.5‰; tank salinity should be 34 ± 2‰.

11.6.26.4.2 Adult topsmelt in each tank are fed twice daily (at 0900 and 1500 hrs) approximately 0.3g of Tetramin™ flake food. Supplemental feedings of krill or chopped squid are recommended. Tanks are siphoned clean once weekly.

11.6.26.4.3 Dyeless yarn spawning substrates are attached to the surface of plastic grids cut from light diffuser panel (7 cm x 10 cm x 1 cm) and weighted to the bottom of each tank. Substrates are checked daily for the presence of eggs.

11.6.26.4.4 Spawning is induced by a combination of three environmental cues: lighting, 'tidal' cycle, and temperature. The photoperiod is 14 hours of light followed by 10 hours of darkness (14L:10D) with lights on at 0600 and off at 2000 hours. Use two cool white 40-watt fluorescent lamps suspended 1.25 meters above the surface of each tank to provide illumination. Light levels at the surface of the tanks should be 12 to 21 $\mu E/m^2/s$.

11.6.26.4.5 A 'tidal signal' of reduced current velocity is produced once daily in each tank, from 2400 to 0200 hrs, by turning off the circulating pump (Middaugh and Hemmer, 1984). A 1500-watt immersion heater is used to maintain constant temperature at 18EC and to provide temperature spikes. For spiking, the temperature is raised from 18EC to 21EC over a 12 h period, then allowed to return to 18EC overnight. The temperature should be checked to the nearest 0.1EC at 1 to 4 hour intervals on days when the temperature spikes are introduced. It is common for the fish to appear stressed during the temperature increase and one or two fish may die. If significant mortality begins to occur, the temperature should be lowered immediately. Significant egg production usually begins within five days of the temperature spike (Middaugh, et al., 1992).

11.6.26.5 Culture Materials

11.6.26.5.1 See Section 5, Facilities and Equipment, for a discussion of suitable materials to be used in laboratory culture of topsmelt. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before re-use.

11.6.26.6 Test Organisms

11.6.26.6.1. Newly fertilized embryos should be placed in screen tubes set in aquaria and equipped with gently flowing seawater at 20 ± 1EC. The embryos can be left attached to the spawning substrates but care should be taken to ensure the substrates are relatively clean and free of food; strands of embryos should not overlap each other on the substrates, and gentle aeration must be provided. Beginning about day 9, check the screen tubes daily for the presence of larvae. Isolate newly-hatched larvae into a separate screen-tube at 21EC by slow siphoning. Provide larvae with newly-hatched *Artemia* nauplii (in excess) at 24-h post-hatch; supply gently flowing seawater, and aeration. Larvae aged 9 to 15 days are used in toxicity tests (McNulty et al., 1994). For information regarding topsmelt larva suppliers call the Marine Pollution Studies Laboratory (408) 624-0947.

11.6.26.6.2 Larvae can be transported in 1-liter ziplock plastic bags (double-bagged). No more than approximately 100 larvae should be transported in any one bag; do not include food. The seawater in the bags should be aerated with pure oxygen for 30 seconds prior to introduction of the larvae. The bag should be packed in an ice chest with one or two blue ice blocks (insulated by newspaper) for transport. The temperature during transport should be held between 15 and 18EC. Larvae should be shipped via air-express overnight couriers.

11.6.26.6.3 Topsmelt larvae can tolerate a relatively wide range of salinities (5 to $35‰) if adequate acclimation is provided (Anderson, et al., In Press). In situations where the test salinity is significantly lower than the salinity at which the larvae were cultured, it may be necessary to acclimate the larvae to the test salinity.

11.7 **EFFLUENTS AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE**

11.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.8 **CALIBRATION AND STANDARDIZATION**

11.8.1 See Section 4, Quality Assurance

11.9 **QUALITY CONTROL**

11.9.1 See Section 4, Quality Assurance

11.10 **TEST PROCEDURES**

11.10.1 TEST DESIGN

11.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

11.10.1.2 Effluent concentrations are expressed as percent effluent.

11.10.2 TEST SOLUTIONS

11.10.2.1 Receiving waters

11.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL would require approximately 1 L of sample per test per day.

11.10.2.2 Effluents

11.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

11.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

11.10.2.2.3 The volume in each test chamber is 200 mL.

11.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

11.10.2.3 Dilution Water

11.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

11.10.2.4 Reference Toxicant Test

11.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

11.10.2.4.2 The preferred reference toxicant for topsmelt is copper chloride ($CuCl_2 \circ 2H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2 \circ 2H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

11.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 56, 100, 180, and 320 µg/L total copper. Prepare one liter of each concentration by adding 0, 5.6, 10.0, 18.0, and 32.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

11.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

11.10.3 START OF THE TEST

11.10.3.1 Prior to Beginning the Test

11.10.3.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section, 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

11.10.3.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (20 ± 1EC) and maintained at that temperature during the addition of dilution water.

11.10.3.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (20 ± 1EC).

11.10.3.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the larvae have been examined at the end of the test.

11.10.3.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

11.10.3.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

11.10.3.2 Randomized Placement of Larvae into Test Chambers

11.10.3.2.1 Larvae must be randomized before placing them into the test chambers. Pool all of the test larvae into a 1-liter beaker by slow siphoning from the screen-tube. The larvae in the screen-tube can be concentrated into the bottom by lifting the tube during siphoning. Using a fire-polished glass tube, place one larva into as many plastic cups as there are test chambers (including reference toxicant chambers). These cups should contain enough reference seawater to maintain water quality and temperature during the transfer process (approx. 50 mL). When each of the cups contains one larva, repeat the process, adding one larva at a time until each cup contains 5 animals.

11.10.3.2.2 Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test larvae. If more than 5 mLs of water are added to the test solution with the juveniles, report the amount on the data sheet. Carefully transfer the larvae into the test chambers immediately after reducing the water volume. Again, make note of any excess dilution of the test solution. Because of the small volumes involved in the transfer process, this is best accomplished in a constant temperature room. Be sure that all water used in culture, transfer, and test solutions is within 1EC of the test temperature.

11.10.3.2.3 Verify that all five animals are transferred by counting the number in each chamber after transfer. This initial count is important because larvae unaccounted for at the end of the test are assumed to be dead.

11.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

11.10.4.1 The light quality and intensity should be at ambient laboratory conditions are generally adequate. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

11.10.4.2 The water temperature in the test chambers should be maintained at 20 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.4.3 The test salinity should be in the range of 5 to 34‰, and the salinity should not vary by more than ± 2‰ among the chambers on a given day. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.4.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

11.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress to the fish.

11.10.6 FEEDING

11.10.6.1 *Artemia* nauplii are prepared as described below.

11.10.6.2 The test larvae are fed newly-hatched (less than 24-h-old) *Artemia* nauplii once a day from Day 0 through Day 6; larvae are not fed on Day 7. Equal amounts of *Artemia* nauplii must be fed to each replicate test chamber to minimize the variability of larval weight. Add 40 newly hatched *Artemia* nauplii per larva twice daily: once in the morning and once in the afternoon. The density of *Artemia* may be determined by pipetting a known volume of nauplii onto a piece of filter paper and counting the number using a dissecting microscope. Feeding excessive amounts of *Artemia* nauplii will result in a depletion in DO to below an acceptable level. Siphon as much of the uneaten *Artemia* nauplii as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

11.10.7 DAILY CLEANING OF TEST CHAMBERS

11.10.7.1 Before the daily renewal of test solutions, uneaten and dead brine shrimp, dead larvae, and other debris are removed from the bottom of the test chambers with a siphon hose. Because of their small size during the first few days of the test, larvae are easily drawn into a siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the live larvae caught up in the siphon can be retrieved, and returned by pipette to the appropriate test chamber and noted on the data sheet.

11.10.8 OBSERVATIONS DURING THE TEST

11.10.8.1 Routine Chemical and Physical Observations

11.10.8.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

11.10.8.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

11.10.8.1.3 Record all the measurements on the data sheet.

11.10.8.2 Routine Biological Observations

11.10.8.2.1 The number of live larvae in each test chamber are recorded daily and the dead larvae are discarded. These data provide daily mortality rates which may be used to calculate 24, 48, and 96-h LC50s.

11.10.8.2.2 Protect the larvae from unnecessary disturbances during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed at all times during the performance of the above operations.

11.10.9 TEST SOLUTION RENEWAL

11.10.9.1 The test solutions are renewed daily using freshly prepared solutions, immediately after cleaning the test chambers. The old solution is carefully siphoned out, leaving enough water so that all of the larvae can still swim freely (approximately 50 mL). Siphon from the bottom of the test chambers so that dead *Artemia* nauplii are removed with the old test solution. It is convenient to siphon old solutions into a small (~500 mL) container in order to ensure that no larvae have been inadvertently removed during solution renewals. If a larva is siphoned, return it to the test chamber and note it on the data sheet.

11.10.9.2 New solution is siphoned into the test chambers using a U-shaped glass tube attached to plastic tubing to minimize disturbance to the larvae.

11.10.9.3 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 4EC. Plastic containers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.10.9.4 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 20 ± 1EC to prepare the test solutions. A sufficient quantity of effluent should be warmed to make daily test solutions.

11.10.10 TERMINATION OF THE TEST

11.10.10.1 Ending the Test

11.10.10.1.1 Record the time the test is terminated.

11.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

11.10.10.2 Sample Preservation

11.10.10.2.1 The surviving larvae in each test chamber (replicate) are counted, and immediately prepared as a group for dry weight determination, or are preserved in 4% formalin then 70% ethanol. Preserved organisms are dried and weighed within 7 d. For safety, formalin should be used under a hood. Note: Death is defined as lack of response to stimulus such as prodding with a glass rod; dead larvae are generally opaque and curled.

11.10.10.3 Weighing

11.10.10.3.1 For immediate drying and weighing, siphon or pour live larvae onto a 500 µm mesh screen in a large beaker to retain the larvae and allow *Artemia* to be rinsed away. Rinse the larvae with reagent water to remove salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of reagent water.

11.10.10.3.2 Small aluminum weighing pans can be used to dry and weigh larvae. An appropriate number of aluminum weigh pans (one per replicate) are marked for identification and weighed to 0.01 mg, and the weights are recorded on the data sheets.

11.10.10.3.3 Immediately prior to drying, the preserved larvae are in reagent water. The rinsed larvae from each test chamber are transferred, using forceps, to a tared weighing pans and dried at 60EC for 24 h, or at 105EC for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing pans containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate. Record the weights.

11.10.10.4 Endpoints

11.10.10.4.1 Divide the dry weight by the number of original larvae (5) per replicate to determine the average dry weight, and record on the data sheets. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 11.11). Complete the summary data sheet after calculating the average measurements and statistically analyzing the dry weights and percent survival for the entire test. Average weights should be expressed to the nearest 0.01 mg.

11.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

11.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE TOPSMELT, *ATHERINOPS AFFINIS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static-renewal
2. Salinity:	5 to 34‰ (± 2‰ of the selected test salinity)
3. Temperature:	20 ± 1EC
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Renewal of test solutions:	Daily
10. Age of test organisms:	9-15 days post-hatch
11. No. larvae per test chamber:	5
12. No. replicate chambers per concentration:	5
13. Source of food:	Newly hatched *Artemia* nauplii
14. Feeding regime:	Feed 40 nauplii per larvae twice daily (morning and night)

15. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
16. Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
17. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
18. Test concentrations:	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
19. Dilution factor:	Effluents: $0.5 Receiving waters: None, or $0.5
20. Test duration:	7 days
21. Endpoints:	Survival and growth (weight)
22. Test acceptability criteria:	$80% survival in controls, 0.85 mg average weight of control larvae (9 day old), LC50 with copper must be #205 µg/L, <25% MSD for survival and <50% MSD for growth

23. Sampling requirement:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected on days one, three, and five with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
24. Sample volume required:	2 L per day

11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 Tests results are acceptable only if all the following requirements are met:

(1) The mean survival of larvae must be at least 80% in the controls.

(2) If the test starts with 9 day old larvae, the mean weight per larva must exceed 0.85 mg in the reference and brine controls; the mean weight of preserved larvae must exceed 0.72 mg.

(3) The LC50 for survival must be within two standard deviations of the control chart mean for the laboratory. The LC50 for survival with copper must be <205 µg/L.

(4) The minimum significant difference (%MSD) of <25% relative to the control for survival for the reference toxicant test. The (%MSD) of <50% relative to the control for growth for the reference toxicant test.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is listed in Table 4.

11.13.1.2 The endpoints of toxicity tests using the topsmelt larvae are based on the adverse effects on survival and growth. The LC50 and the IC25 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981)(see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50 and IC25. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50 and IC25. See the Appendices for examples of the manual computations and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

11.13.2 EXAMPLE OF ANALYSIS OF TOPSMELT, *ATHERINOPS AFFINIS* SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 1 and 2. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoints.

11.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's

Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

11.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method may be used to estimate the LC50 (see Appendices H-K).

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Topsmelt Larval Survival and Growth Test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix . The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. A plot of the survival proportions is provided in Figure 5. Since there was 100% mortality in all five replicates for the 100 µg/L and 180 µg/L concentrations, they are not included in the statistical analysis and are considered qualitative mortality effects.

TABLE 4. SUMMARY OF SURVIVAL AND GROWTH DATA FOR TOPSMELT, *ATHERINOPS AFFINIS*, LARVAE EXPOSED TO COPPER FOR SEVEN DAYS[1]

Copper Conc. (µg/L)	Replicate Survival Proportions A	B	C	D	E	Mean Proportion Survival
0.0	1.0	0.8	1.0	1.0	1.0	0.96
32.0	1.0	1.0	1.0	1.0	1.0	1.00
56.0	0.0	0.6	0.2	1.0	0.6	0.48
100.0	0.0	0.0	0.0	0.0	0.0	0.00
180.0	0.0	0.0	0.0	0.0	0.0	0.00

Conc. (µg/L)	Replicate Average Dry Weights (mg) A	B	C	D	E	Mean Dry Wgt (mg)
0.0	0.00134	0.00153	0.00134	0.00146	0.00144	0.00142
32.0	0.00146	0.00142	0.00150	0.00138	0.00128	0.00141
56.0	--	0.00147	0.00170	0.00124	0.00130	0.00114
100.0	--	--	--	--	--	--
180.0	--	--	--	--	--	--

[1]Five replicates of 5 larvae each.

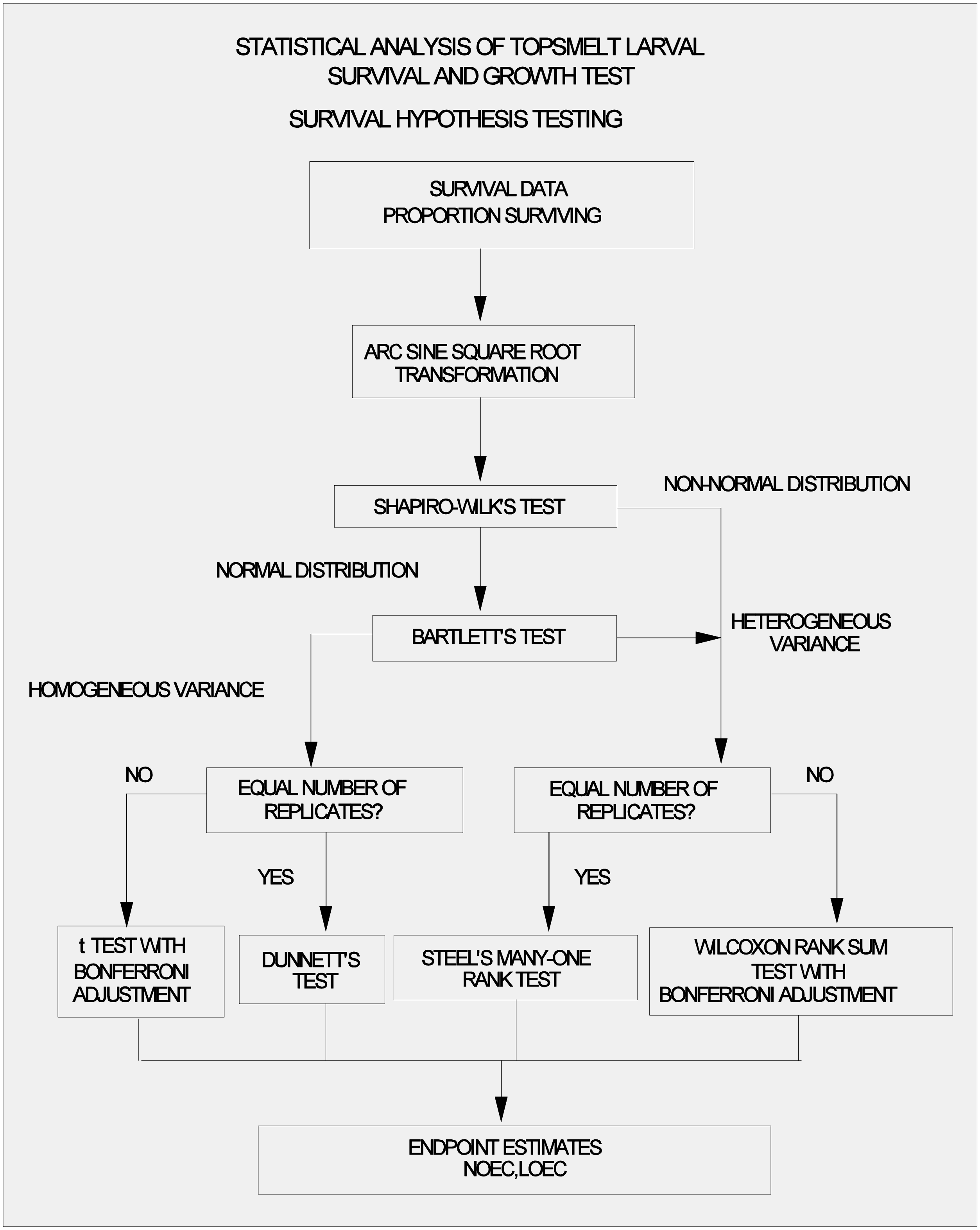

Figure 1. Flowchart for statistical analysis of the topsmelt, *Atherinis affinis*, larval survival data by hypothesis testing.

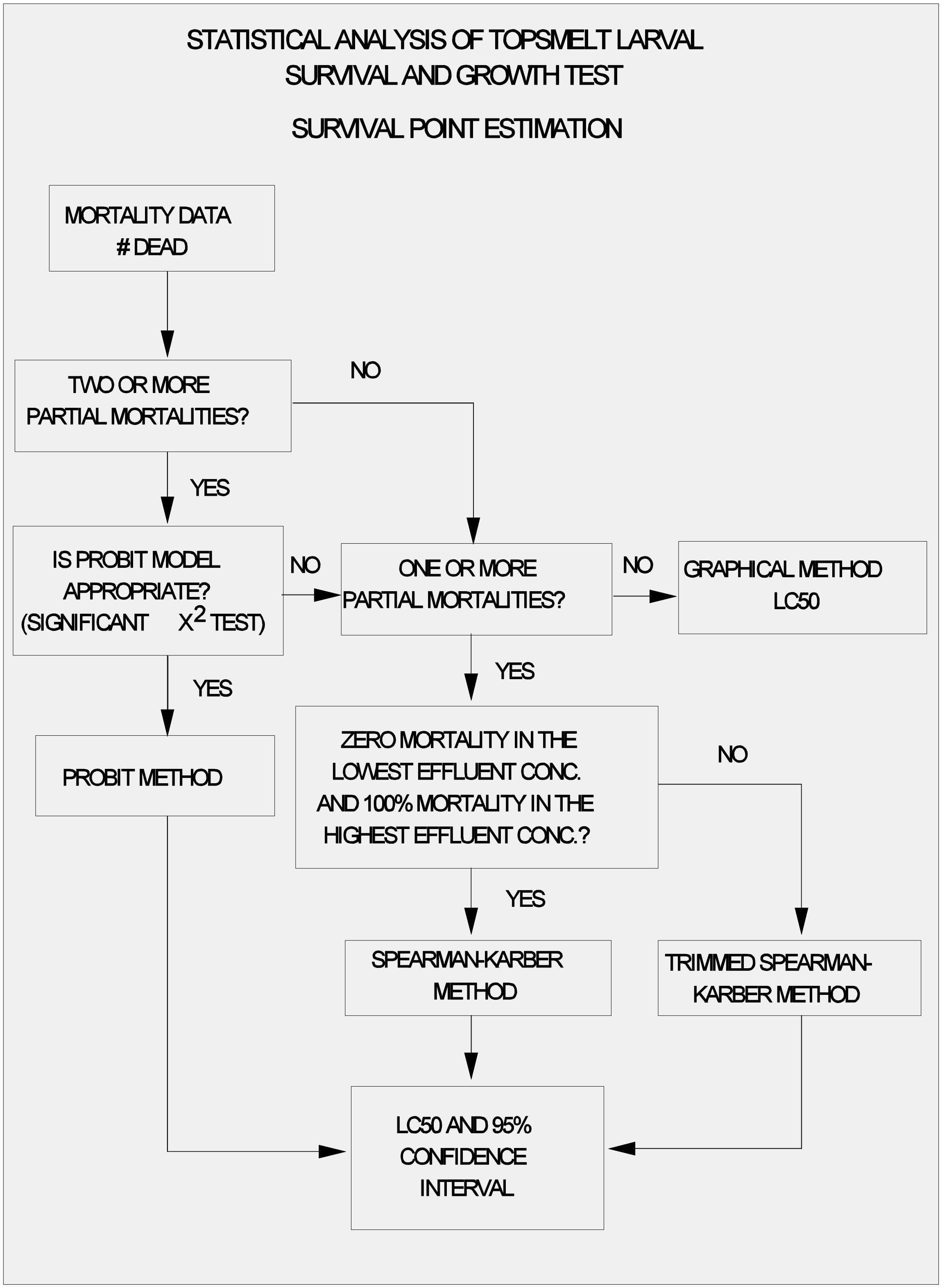

Figure 2. Flowchart for statistical analysis of the topsmelt, *Atherinis affinis*, larval survival data by point estimation.

TABLE 5. TOPSMELT, *ATHERINOPS AFFINIS*, SURVIVAL DATA

	Replicate	Control	Copper Concentration (µg/L) 32.0	56.0
RAW	A	1.0	1.0	0.0
	B	0.8	1.0	0.6
	C	1.0	1.0	0.2
	D	1.0	1.0	1.0
	E	1.0	1.0	0.6
ARC SINE SQUARE ROOT TRANSFORMED	A	1.345	1.345	0.225
	B	1.107	1.345	0.886
	C	1.345	1.345	0.464
	D	1.345	1.345	1.345
	E	1.345	1.345	0.886
Mean ($\bar{Y}_i$)		1.297	1.345	0.761
S^2		0.0113	0.000	0.187
i^i		1	2	3

11.13.2.6 Test for Normality

11.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

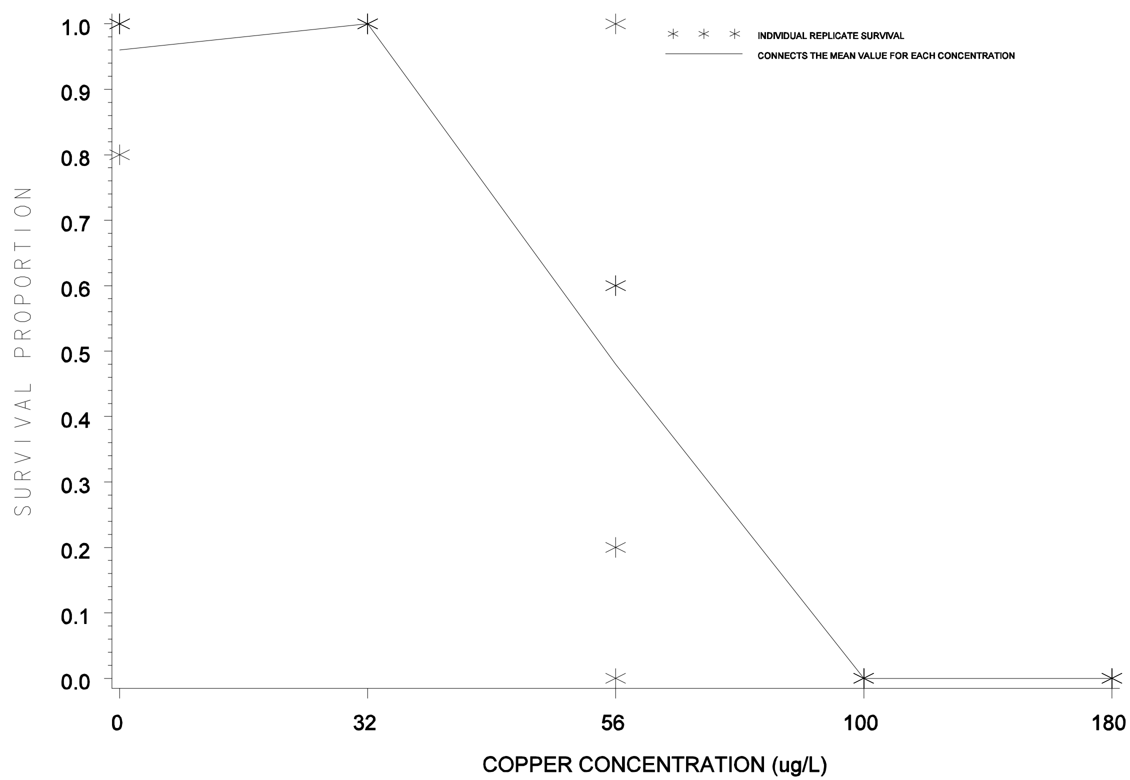

Figure 3 Plot of mean survival proportion data in Table 5.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Copper Concentration (µg/L)	
		32.0	56.0
A	0.048	0.000	-0.536
B	-0.190	0.000	0.125
C	0.048	0.000	-0.297
D	0.048	0.000	0.584
E	0.048	0.000	0.125

11.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

11.13.2.6.3 For this set of data,

$$n = 15$$

$$\overline{X} = \frac{1}{15}(0.003) = 0.000$$

$$D = 0.793$$

11.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.536	9	0.048
2	-0.297	10	0.048
3	-0.190	11	0.048
4	0.000	12	0.048
5	0.000	13	0.125
6	0.000	14	0.125
7	0.000	15	0.584
8	0.000		

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 15 and k = 7. The a_i values are listed in Table 8.

11.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7. For the data in this example,

$$W = \frac{1}{0.793}(0.817)^2 = 0.842$$

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 11.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 15 observations is 0.835. Since W = 0.842 is greater than the critical value, conclude that the data are normally distributed.

11.13.2.6.8 Since the variance of the lowest copper concentration group is zero, Bartlett's test statistic can not be calculated. Therefore, the survival data variances are considered to be heterogeneous.

11.13.2.6.9 Since the data do not meet the assumption of homogeneity of variance, Steel's Many-one Rank Test will be used

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5150	1.120	$X^{(15)} - X^{(1)}$
2	0.3306	0.422	$X^{(14)} - X^{(2)}$
3	0.2495	0.315	$X^{(13)} - X^{(3)}$
4	0.1878	0.048	$X^{(12)} - X^{(4)}$
5	0.1353	0.048	$X^{(11)} - X^{(5)}$
6	0.0880	0.048	$X^{(10)} - X^{(6)}$
7	0.0433	0.048	$X^{(9)} - X^{(7)}$

to analyze the survival data.

11.13.2.7 Steel's Many-one Rank Test

11.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

11.13.2.7.2 An example of assigning ranks to the combined data for the control and 32.0 µg/L copper concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are next summed for each copper concentration, as shown in Table 11.

11.13.2.7.3 For this example, determine if the survival in any of the copper concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the survival at each of the various copper concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with two concentrations (excluding the control) and five replicates is 18 (see Table 5, Appendix E).

11.13.2.7.4 Since the rank sum for the 56.0 µg/L copper concentration is equal to the critical value, the proportion surviving in the 56.0 µg/L concentration is considered significantly less than that in the control. Since the other rank sum is not less than or equal to the critical value, it is not considered to have a significantly lower proportion surviving than the control. Hence, the NOEC and the LOEC are the 32.0 µg/L and 56.0 µg/L concentrations, respectively.

11.13.2.8 Calculation of the LC50

11.13.2.8.1 The data used for the calculation of the LC50 is summarized in Table 12. For estimating the LC50, the data for the 100 µg/L and 180 µg/L copper concentrations with 100% mortality are included.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 32.0 µg/L COPPER CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Surviving	Copper Concentration (µg/L)
1	1.107	Control
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control

TABLE 10. TABLE OF RANKS

Replicate	Control	Copper Concentration (µg/L) 32.0	Copper Concentration (µg/L) 56.0
A	1.345 (6, 8)	1.345 (6)	0.225 (1)
B	1.107 (1, 5)	1.345 (6)	0.886 (3.5)
C	1.345 (6, 8)	1.345 (6)	0.464 (2)
D	1.345 (6, 8)	1.345 (6)	1.345 (8)
E	1.345 (6, 8)	1.345 (6)	0.886 (3.5)

TABLE 11. RANK SUMS

Copper Concentration (µg/L)	Rank Sum
32.0	30
56.0	18

11.13.2.8.2 Because there are is only one partial mortality in the set of copper concentration responses, Probit Analysis is not appropriate to calculate the LC50 and 95% confidence interval for this set of test data. Inspection of the data reveals that, once the data is smoothed and adjusted, the proportion mortality in the lowest effluent concentration will be zero and the proportion mortality in the highest effluent concentration will be one. Therefore, the Spearman-Karber Method is appropriate for this data.

11.13.2.8.3 Before the LC50 can be calculated the data must be smoothed and adjusted. For the data in this example, because the observed proportion mortality for the 32.0 µg/L copper concentration is less than the observed response proportion for the control, the observed responses for the control and this group must be averaged:

$$p_0^s = p_1^s = \frac{0.040+0.000}{2} = 0.020$$

Where: p_i^s = the smoothed observed mortality proportion for effluent concentration i.

11.13.2.8.3.1 Because the rest of the responses are monotonic, additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table 12.

11.13.2.8.4 Because the smoothed observed proportion mortality for the control is now greater than zero, the data in each effluent concentration must be adjusted using Abbott's formula

(Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i

11.13.2.8.4.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.020 - 0.020}{1 - 0.020} = \frac{0.000}{0.980} = 0.0$$

$$p_2^a = \frac{p_2^s - p_0^s}{1 - p_0^s} = \frac{0.520 - 0.020}{1 - 0.020} = \frac{0.500}{0.980} = 0.510$$

$$p_3^a = p_4^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.020}{1 - 0.020} = \frac{0.980}{0.980} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table 12.

11.13.2.8.5 Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a - p_i)(X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control

TABLE 13. TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH DATA

Replicate	Control	Copper Concentration (µg/L) 32.0	56.0	100.0	180.0
A	0.00134	0.00146	-	-	-
B	0.00153	0.00142	-	-	-
C	0.00134	0.00150	-	-	-
D	0.00146	0.00128	-	-	-
E	0.00144	0.00141	-	-	-
Mean($\bar{Y}_i$)	0.00142	0.00141	-	-	-
S_i^2	0.000000006	0.000000007	-	-	-
i	1	2	3	4	5

11.13.2.8.5.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$
\begin{aligned}
m = {} & [(0.510 - 0.000)\ (1.5051 + 1.7482)]/2\ + \\
& [(1.000 - 0.510)\ (1.7482 + 2.0000)]/2\ + \\
& [(1.000 - 1.000)\ (2.0000 + 2.2553)]/2\ + \\
\\
= {} & 1.7479
\end{aligned}
$$

11.13.2.8.6 Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a (1-p_i^a)(X_{i+1}-X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.6.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$V(m) = (0.510)(0.490)(2.0000 - 1.5051)^2/4(24) + (1.000)(0.000)(2.2553 - 1.7482)^2/4(24)$$

$$= 0.0006376$$

11.13.2.8.7 Calculate the 95% confidence interval for m: m ± 2.0 %&&&&&

11.13.2.8.7.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.7479 \pm 2\sqrt{0.0006376} = (1.6974, 1.7984)$$

11.13.2.8.8 The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking $base_{10}$ antilogs of the above values.

11.13.2.8.8.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.7479) = 56.0 µg/L.

11.13.2.8.8.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.6974) = 49.8 µg/L

upper limit: antilog(1.7984) = 62.9 µg/L

11.13.3 EXAMPLE OF ANALYSIS OF TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 4.

The response used in the statistical analysis is mean weight per surviving organism for each replicate. The IC25 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis testing consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steels' Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

11.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 13. A plot of the mean weights for each treatment is provided in Figure 5. Since there is no survival in the 100 µg/L and 180 µg/L copper concentrations, they are not considered in the growth analysis. Additionally, since there is significant mortality in the 56.0 µg/L concentration, its effect on growth is not considered.

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 14.

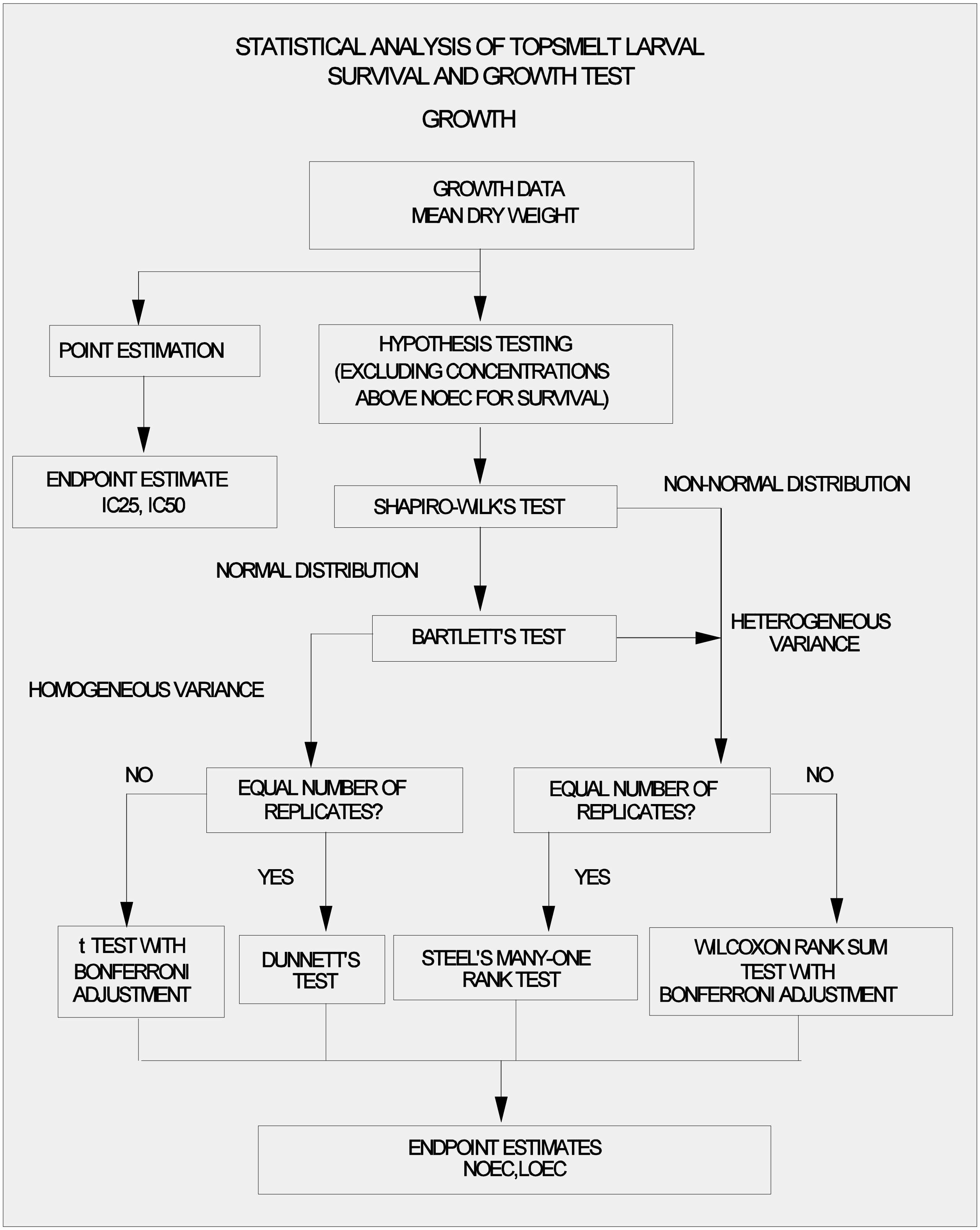

Figure 4. Flowchart for statistical analysis of the topsmelt, *Atherinops affinis*, larval growth data.

TABLE 13. TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH DATA

		Copper Concentration (µg/L)			
Replicate	Control	32.0	56.0	100.0	180.0
A	0.00134	0.00146	-	-	-
B	0.00153	0.00142	-	-	-
C	0.00134	0.00150	-	-	-
D	0.00146	0.00128	-	-	-
E	0.00144	0.00141	-	-	-
Mean($\bar{Y}_i$)	0.00142	0.00141	-	-	-
S_i^2	0.000000006	0.000000007	-	-	-
i	1	2	3	4	5

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	32.0 µg/L Copper
A	-0.00008	0.00005
B	0.00011	0.00001
C	-0.00008	0.00009
D	0.00004	-0.00003
E	0.00002	-0.00013

11.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

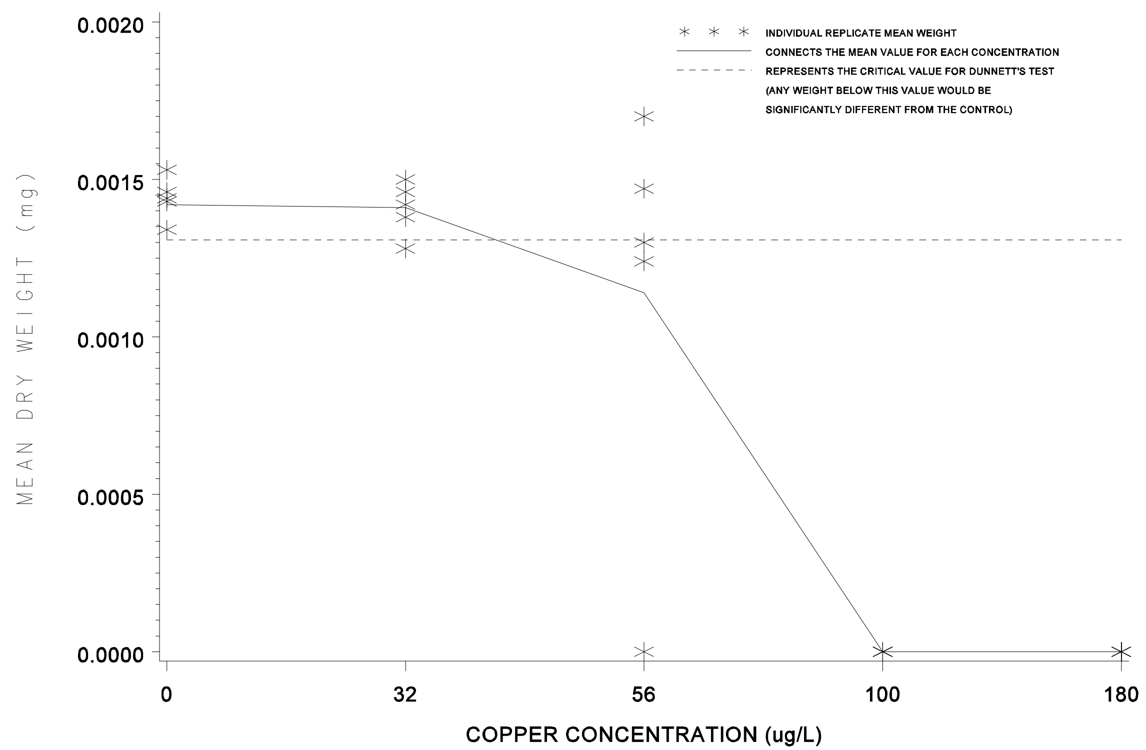

Figure 5. Plot of weight data from topsmelt, *Atherinis affinis*, larval survival and growth test.

For this set of data, n = 10

$$\& = \frac{1}{10}(0.00) = 0.00$$

$$D = 0.000000055$$

11.13.3.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 15.

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 10 and k = 5. The a_i values are listed in Table 16.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.00013	6	0.00002
2	-0.00008	7	0.00004
3	-0.00008	8	0.00005
4	-0.00003	9	0.00009
5	0.00001	10	0.00011

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5739	0.00024	$X^{(10)} - X^{(1)}$
2	0.3291	0.00017	$X^{(9)} - X^{(2)}$
3	0.2141	0.00013	$X^{(8)} - X^{(3)}$
4	0.1224	0.00007	$X^{(7)} - X^{(4)}$
5	0.0399	0.00001	$X^{(6)} - X^{(5)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{0.000000055}(0.0002305)^2 = 0.966$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 10 observations (n) is 0.781. Since W = 0.966 is greater than the critical value, the conclude that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

11.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{p} V_i\right) \ln \bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i

p = number of levels of effluent concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}\left[\sum_{i=1}^{p} 1/V_i - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

11.13.3.6.2 For the data in this example (see Table 14), all effluent concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(8)\ln(6.5 \times 10^{-9}) - 4\sum_{i=1}^{p} \ln(S_i^2)]/1.125$$

$$= [8(-18.851) - 4(-37.709)]/1.125$$

$$= 0.028/1.125$$

$$= 0.0249$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with one degree of freedom, is 6.635. Since B = 0.0249 is less than the critical value of 6.635, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{p} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = 5$

$N = 10$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 0.00711$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 0.00704$

$G = T_1 + T_2 = 0.01415$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(1.001137 \times 10^{-4}) - \frac{(0.01415)^2}{10} = 4.90 \times 10^{-10}$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0000201 - \frac{(0.01415)^2}{10} = 7.775 \times 10^{-8}$$

$$SSW = SST - SSB = 7.775 \times 10^{-8} - (4.9 \times 10^{-10}) = 7.726 \times 10^{-8}$$

$$S_B = SSB/(p-1) = (4.9 \times 10^{-10})/(2-1) = 4.9 \times 10^{-10}$$

$$S_W = SSW/(N-p) = 7.726 \times 10^{-8}/(10-2) = 9.658 \times 10^{-9}$$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	1	4.90×10^{-10}	4.9×10^{-10}
Within	8	7.726×10^{-8}	9.658×10^{-9}
Total	9	7.775×10^{-8}	

11.13.3.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w \sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean dry weight for effluent concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example there is only one comparison, of the 32.0 µg/L copper concentration with the control. The calculation is as follows:

$$t_2 = \frac{(0.00142 - 0.00141)}{[\,9.828 \times 10^{-5}\sqrt{(1/5)+(1/5)}\,]} = 0.161$$

TABLE 19. CALCULATED t VALUES

Copper Concentration (µg/L)	i	t_i
32.0	2	0.161

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 8 degrees of freedom for error and one concentration (excluding the control) the critical value is 1.86. The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_2 is less than 1.86, the 32.0 µg/L concentration does not have significantly lower growth than the control. Hence the NOEC and the LOEC for growth cannot be calculated.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_w = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 1.86\,(9.828 \times 10^{-5})\sqrt{(1/4) + (1/4)}$$

$$= 1.86\ (9.828 \times 10^{-5})(0.632)$$

$$= 0.000116$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.000116 mg.

11.13.3.7.10 This represents a 8.2% reduction in mean weight from the control.

11.13.3.8 Calculation of the ICp

11.13.3.8.1 The growth data from Table 4 are utilized in this example. As seen from Table 4 and Figure 6, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is less than or equal to the mean response for the previous concentration and the responses between concentrations follow a linear trend). Therefore, the means do not require smoothing prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

11.13.3.8.2 Since $\bar{Y}_5 = 0 < \bar{Y}_4 = 0 < \bar{Y}_3 = 0.00114 < \bar{Y}_2 = 0.00141 < \bar{Y}_1 = 0.00142$, set $M_1 = 0.00142$, $M_2 = 0.00141$, $M_3 = 0.00114$, $M_4 = 0$ and $M_5 = 0$.

11.13.3.8.3 Table 20 contains the response means and smoothed means and Figure 8 gives a plot of the smoothed response curve.

11.13.3.8.4 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.001065 mg, where $M_1(1\text{-}p/100) = 0.00142(1\text{-}25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.001065 mg, is bracketed by $C_3 = 56.0$ µg/L copper and $C_4 = 100.0$ µg/L copper.

11.13.3.8.5 Using the equation from Section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 56.0 + [0.00142(1 - 25/100) - 0.00114]\ \frac{(100.0 - 56.0)}{(0.0 - 0.00114)}$$

$$= 58.9\ \mu g/L.$$

11.13.3.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 58.9089 µg/L. The empirical 95% confidence interval for the true mean was 44.2778 µg/L to 67.0000 µg/L. The computer program output for the IC25 for this data set is shown in Figure 7.

TABLE 20. TOPSMELT, *ATHERINOPS AFFINIS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means (mg) $\bar{Y}_i$	Smoothed Means (mg) M_i
Control	1	0.00142	0.00142
32.0	2	0.00141	0.00141
56.0	3	0.00114	0.00114
100.0	4	0.0	0.0
180.0	5	0.0	0.0

11.14.1 PRECISION

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Data on the single-laboratory precision of the topsmelt larval survival and growth test using copper chloride as the reference toxicant are provided in Tables 21 and 22. In the five copper tests presented here, the NOECs for survival were 100 µg/L for all tests but one; this test had a NOEC of 180 µg/L. The coefficient of variation for copper based on the LC25 is 17.3% for survival; the coefficient of variation for copper based on the LC50 is 9.7% for survival. The weight endpoint was less sensitive than survival in all but one test. An IC25 could be calculated for three of five tests and the coefficient of variation for these three tests was 60.69%, the coefficient of variation based on the IC50 for these three tests was 4.75%.

11.14.1.2 Multilaboratory Precision

14.11.1.2.1 Data on the interlaboratory precision of the topsmelt larval survival and growth test are provided in Table 23. Three separate interlaboratory tests were conducted. In the first comparison both laboratories derived identical NOECs for copper (100µg/L). The coefficient of variation, based on LC50s for survival was 36%. In the second comparison the NOEC for effluent was 20% at both laboratories. The coefficient of variation, based on the LC50s for survival was 19%. In the third comparison the NOEC for copper was 32 µg/L at both laboratories. The coefficient of variation, based on the LC50s for survival was 3%.

11.11.2 ACCURACY

11.11.2.1 The accuracy of toxicity tests cannot be determined.

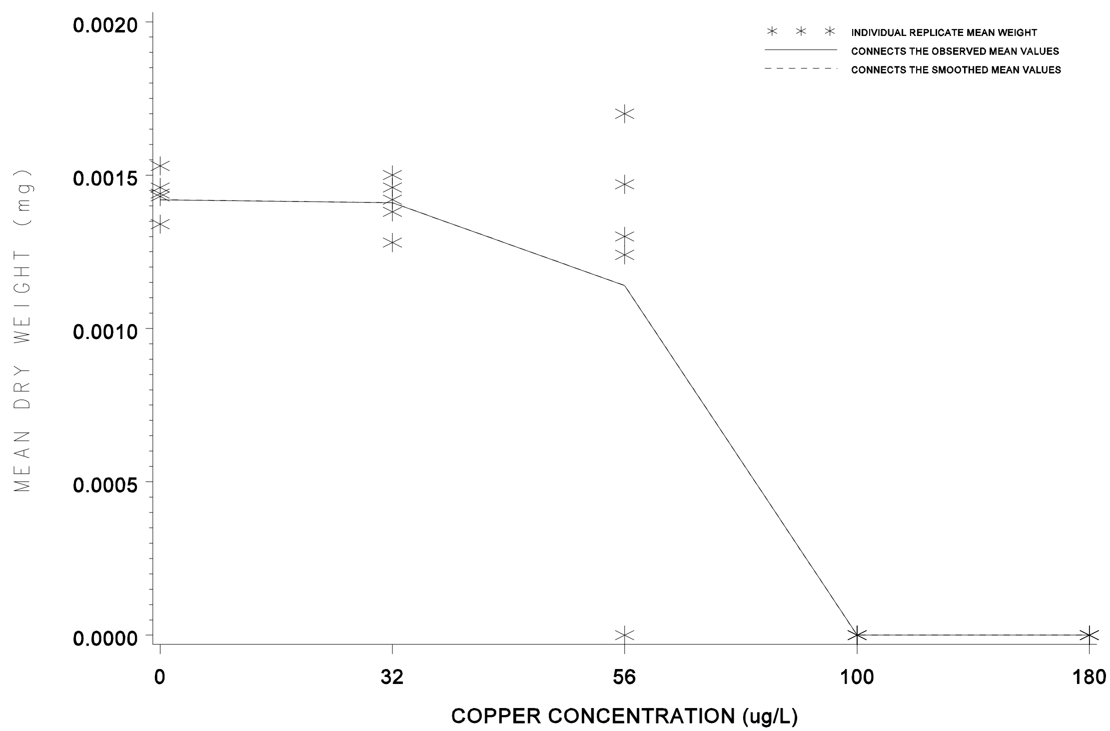

Figure 6. Plot of raw data, observed means, and smoothed means for topsmelt, *Atherinis affinis*, growth data from Tables 4 and 21.

Conc. ID	1	2	3	4	5
Conc. Tested	0	32	56	100	180
Response 1	.00134	.00146	0	0	0
Response 2	.00153	.00142	.00147	0	0
Response 3	.00134	.00150	.00170	0	0
Response 4	.00146	.00138	.00124	0	0
Response 5	.00144	.00128	.00130	0	0

```
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Atherinops affinis
Test Duration:          7 days
DATA FILE: wc_aa.icp
OUTPUT FILE: wc_aa.i25
```

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.001	0.000	0.001
2	5	32.000	0.001	0.000	0.001
3	5	56.000	0.001	0.001	0.001
4	5	100.000	0.000	0.000	0.000
5	5	180.000	0.000	0.000	0.000

```
The Linear Interpolation Estimate:    58.9089   Entered P Value: 25
--------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  58.1571 Standard Deviation:     7.9299
Original Confidence Limits:   Lower:    44.2778 Upper:    67.0000
Expanded Confidence Limits:   Lower:    36.9622 Upper:    71.0455
Resampling time in Seconds:     0.11  Random_Seed: -498847050
```

Figure 7. ICPIN program output for the IC25

TABLE 21. SINGLE LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS* SURVIVAL ENDP0INT WITH COPPER (CU FG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	NOEC	LC25	LC50
1	100	142.1	187.4
2	100	NC[3]	162.4
3	100	151.7	165.6
4	180	181.0	190.6
5	100	119.2	204.0

# of Tests	Statistic	LC25	LC50
5	Mean SD CV (%)	148.5 25.6 17.3	182.0 17.6 9.7%

TABLE 22. SINGLE LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS* GROWTH ENDP0INT WITH COPPER (CU FG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	NOEC	LC25	LC50
1	180	222.1	264.2
2	180	NC[4]	NC[4]
3	>180	NC[4]	NC[4]
4	56	47.6	NC[4]
5	>180	NC[4]	NC[4]

# of Tests	Statistic	LC25	LC50
5	Mean SD CV (%)	156.8 95.2 60.7%	

[1]Data from Anderson et al. 1994; point estimates calculated using probit analysis, except where noted.
[2]Five replicate exposure chambers with five larvae per chamber were used for each treatment.
[3]LC50 calculated using Spearman-Karber method, this method does not calculate an LC25.
[4]Point estimate not calculated because the response was less than either 25 or 50%.

TABLE 23. MULTI-LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH AND SURVIVAL TEST CONDUCTED WITH COPPER (CU FG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	Toxicant	Laboratory	Survival		Growth
			NOEC	LC50	
1	Copper[a]	1[b]	100	162.0	NS[c]
	Copper[a]	2[d]	100	274.0	NS
	CV		36%		
2	Effluent	1[b]	20	31.4	NS
	Effluent	2[e]	20	23.9	10
	CV		19%		
3	Copper[a]	1[b]	32	55.7	NS
	Copper[a]	1[e]	32	58.4	NS
	CV		3%		

Two separate interlaboratory comparisons were conducted, in August 1990 and August 1991.

[a]The August 1990 copper test was conducted at 34‰ salinity; the August 1991 copper test was conducted at 20‰ salinity.
[b]Marine Pollution Studies Laboratory, Monterey County, California.
[c]Not Significant.
[d]Vantuna Research Group, Occidental College, California.
[e]Chevron Research and Technology Co., Environmental Research Group.

APPENDIX I. TOPSMELT TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 g of copper chloride ($CuCl_2 \circ 2H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 56, 100, 180, and 180 µg/L by adding 0, 5.6, 10.0, 18.0, and 32.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 20EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain 9-15 day old larvae from a commerical supplier or in-house cultures.

B. Larvae must be randomized before placing them into the test chambers. Be sure that all water used in culture, transfer, and test solutions is within 1EC of the test temperature.

C. Remove all dead larvae daily, and add 40 newly hatched *Artemia* nauplii per larva twice daily; once in the morning and once in the afternoon. Adjust feeding to account for larva mortality.

D. Renew test solutions daily using freshly prepared solutions, immediately after cleaning the test chambers.

E. After 7 days, count and record the number of live and dead larvae in each chamber. After counting, use the randomization sheet to assign the correct test concentration to each chamber. Remove all dead larvae.

F. The surviving larvae in each test chamber are immediately prepared as a group for dry weight determination, or preserved in 4% formalin then 70% ethanol. Preserved organisms are dried and weighed with 7 days.

G. Carefully transfer the larvae to a prenumbered, preweighed micro-weigh boat using fine-tipped forceps. Dry for 24 hours at 60EC or at 105EC for a minimum of 6 hours. Weigh each weigh boat on a microbalance (accurate to 1 µg). Record the chamber number, larvae weight, weigh boat weight (recorded previously), and number of larvae per weigh boat (replicate) on the data sheet.

H. Analyze the data.

I. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Larval Fish Toxicity Test

Test Start Date: Start Time:
Fish Species:

Test End Date: End Time:
Collection/Arrival Date:

Reference Toxicant:
Broodstock Source:

Fish Age at Start:

Test Cont. #	Concentration	Numer Alive							Total Number Alive	Total Number at Start	Notes
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
Computer Data Storage Disk File											

Note: See larval weight data on separate sheet.

Data Sheet for Weighing Larval Fish

Test Start Date: Start Time: Fish Species :

Test End Date: End Time: Collection/Arrival Date:

Toxicant: Fish Age at Start:

Sample Source:

Sample Type: Sediment Elutriate Porewater Water

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (mg)	Total Weight (mg)	Weight of Larval Fish (mg)	Number of Fish Larvae	Weight per Larval Fish (mg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
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33							
34							
35							

Computer Data Storage Notes
Disk:
File:

Note: See larval mortality data on separate sheet.